1993. The Journal of Arachnology 21:194–201

DNA SEQUENCE DATA INDICATES THE POLYPHYLY OF THE FAMILY CTENIDAE (ARANEAE)

Kathrin C. Huber[1], **Thomas S. Haider**[2], **Manfred W. Müller**[2], **Bernhard A. Huber**[1], **Rudolf J. Schweyen**[2], and **Friedrich G. Barth**[1]: [1]Institut für Zoologie, Althanstr. 14; 1090 Wien; and [2]Institut für Mikrobiologie und Genetik; Dr. Bohrgasse 9; 1030 Wien (Vienna), Austria.

ABSTRACT. Mitochondrial DNA fragments comprising more than 400 bases of the 16S rDNA from nine spider species have been sequenced: *Cupiennius salei*, *C. getazi*, *C. coccineus* and *Phoneutria boliviensis* (Ctenidae), *Pisaura mirabilis*, *Dolomedes fimbriatus* (Pisauridae), *Pardosa agrestis* (Lycosidae), *Clubiona pallidula* (Clubionidae) and *Ryuthela nishihirai* (syn. *Heptathela nishihirai*; Heptathelidae: Mesothelae). Sequence divergence ranges from 3–4% among *Cupiennius* species and up to 36% in pairwise comparisons of the more distantly related spider DNAs. Maximally parsimonious gene trees based on these sequences indicate that *Phoneutria* and *Cupiennius* are the most distantly related species of the examined Lycosoidea. The monophyly of the family Ctenidae is therefore doubted; and a revision of the family, which should include DNA-data, is needed.

Cupiennius salei (Ctenidae) is one of the most extensively studied species of spiders (see Lachmuth et al. 1985). The phylogeny of the Ctenidae, a mainly South and Central American family, is poorly understood; and systematists propose highly contradicting views on its classification and phylogenetic placement (see e. g., Lehtinen 1967; Bücherl 1969). Coddington & Levi (1991) have recently questioned the monophyly of the Ctenidae, showing that the available information about taxonomically useful characters is still meager and fragmentary.

In recent years the value of DNA sequence data for taxonomic and phylogenetic research has become increasingly clear. DNA sequences contain a nearly inexhaustable quantity of information and may provide valuable insight allowing the evaluation of groups whose phylogeny is largely unresolved by morphological and other data (Kocher et al. 1989; Gatesy et al. 1992; Cunningham et al. 1992; review: Fernholm et al. 1989). Moreover, sequencing DNA—above all mitochondrial DNA (mtDNA; mainly maternal inheritance, lack of recombination)—has specific advantages over other techniques of genetic comparisons such as DNA/DNA hybridization or isoenzyme analysis: e. g., greater resolving power over a hierarchical range of intraspecific to intergeneric comparison and easy comparability with sequences from other species (see also Wilson et al. 1985). The polymerase chain reaction (PCR, Mullis & Faloona 1987; Saiki et al. 1988) is a fast alternative to conventional cloning to get a high copy number of the DNA segment of interest. The PCR depends on the availability of oligonucleotides that specifically bind to the flanking sequences of this DNA segment. These oligonucleotides serve as primers for a polymerization reaction that copies the segment *in vitro*. The PCR-product obtained is suitable for direct sequencing.

The principal aim of the present study was to elucidate the phylogenetic position of the family Ctenidae within the Lycosoidea *sensu* Homann (1971), using four ctenids, two pisaurids, one lycosid, one clubionid and one liphistiomorph spider. Specific PCR-products were obtained by using primers for the mitochondrial 16S ribosomal DNA, designed according to those used by Cunningham et al. (1992). The molecular data largely agree with the conclusions drawn from morphological taxonomy. The most intriguing and surprising result of our study is the indication of a considerable phylogenetic distance between *Cupiennius* and *Phoneutria*.

METHODS

Animals and DNA extraction.—Table 1 lists the animals investigated in this study. In the bigger spiders, muscle tissue was dissected out of the femora; for the smaller ones, the complete prosoma and legs were used to extract DNA. Tissues were put into a digestive solution (70 mM NaCl, 10 mM Tris-HCl pH 7.4, 25 mM EDTA pH 8.0, 0.9% SDS, 6 μg/ml Proteinase K), and incubated for 2–8 h in a water bath at

Table 1.—Systematic position and geographical origin of the spiders investigated. The number of individuals that were sequenced is given in parentheses.

Spider classification	Geographical origin
Mesothelae	
(1) *Ryuthela nishihirai* (Haupt 1979)	Japan
Opisthothelae	
Clubionidae	
(1) *Clubiona pallidula* (Clerck 1757)	Austria
Lycosoidea	
Ctenidae	
(1) *Cupiennius salei* (Keyserling 1876)	Mexico
(2) *Cupiennius getazi* Simon 1891	Costa Rica
(1) *Cupiennius coccineus* F. Pickard-Cambridge 1901	Costa Rica
(2) *Phoneutria boliviensis* (F. Pickard-Cambridge 1897)	Costa Rica
Lycosidae	
(2) *Pardosa agrestis* (Westring 1862)	Austria
Pisauridae	
(2) *Dolomedes fimbriatus* (Clerck 1757)	Austria
(1) *Pisaura mirabilis* (Clerck 1757)	Austria

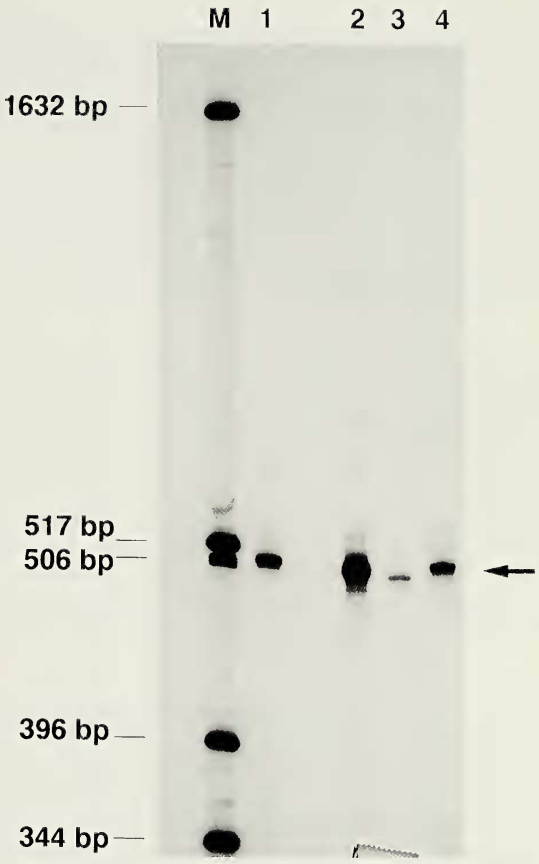

Figure 1.—Products of a radioactively labelled PCR amplification of a mt16S rDNA fragment. M = marker; 1 = *Cupiennius getazi*; 2 = *Pardosa agrestis*; 3 = *Clubiona pallidula*; 4 = *Dolomedes fimbriatus*. Arrow indicates the main products.

50 °C. Proteins were then precipitated with 5 M potassium acetate and RNA was digested by RNAse A (125 μg/ml; 15 min, 37 °C). DNA was precipitated with 60% v/v isopropanol (10 min, −20 °C), pelleted by centrifugation at 15,000 rpm for 20 min at 4 °C, washed with 400 μl of 70% ethanol, dried at 45 °C and resuspended in 20–40 μl TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0). The DNA preparation was stored at −20 °C.

Amplification and sequencing of rDNA.—The mt16S rDNA fragment was amplified using PCR with mt16S rDNA primers as designed by Cunningham et al. (1992), but with terminal extensions for Sac I restriction endonuclease (16sar: 5′–ATAGAGCTCCCATGGCGCCTGTTTATCAAAAACAT-3′ and 16sbr: 5′-ATAGAGCTCCCATGGCCGGTCTGAACTCAGATCACGT-3′). For the amplification assay 90–700 ng DNA and 2.5 units of DNA polymerase from *Thermus aquaticus* (Taq, Stratagene) were incubated in 100 μl of PCR buffer (Stratagene) with each of the four deoxynucleoside triphosphates (50 μM) and the primers (0.4 μM). For radioactive PCR 2-2.5 μCi alpha–^{32}P–dATP was added. The thermal profile for 40 cycles was as follows: (1) DNA melting for 1.5 min at 94 °C, (2) annealing for 2 min at 56 °C and (3) polymerization for 2 min at 72 °C. The product was electrophoresed on a 5% polyacrylamide gel (bisacrylamid : acrylamid 1:30; 8 M urea). Upon autoradiography of the gels the band of expected size was excised and a small piece was used for reamplification in 200 μl of buffer (concentration as above, without alpha–^{32}P–dATP). DNA was purified by the Geneclean II-kit (Bio 101) procedure according to the manufacturer's instruction. The template was then sequenced by the dideoxy chain termination

```
                                                                                                  60
Ryuthela        GTTGGTAATA AAAAATCTTA CCTGCTCCCT GCTATAAGTT AATAGCCGCA GTATTATGAC
Dolomedes       AGAAA.T..T ..T.G.AAA. T......AA. .AA.ATT-.A .........- -ATA------
Pisaura         AGAAA.T..T ..T.G.AAA. T......AA. .A..A.T-.C .........- -AT.------
Pardosa         AGAAT....T ..T...AAAT .......AA. .A-.A.T-.A .........- -.T.------
C.salei         AGAAA....T ..T.G.AAA. T......AA. .A.T..T-.A .........- -.TA------
C.getazi        AGAAA....T ..T.G.AAA. T......AA. .A.T..T-.A .........- -.T.------
C.coccineus     AGAAA....T ..T.G.AAA. T......AA. .A.T..T-.A .........- -.T.------
Phoneutria      A-AAA.T.AT ..T.G.AAG. T......AA. .A-.A.T-.A .......... -ACA------
Clubiona        AGATT.T.AT ..T.G.A.A. T......AA. .AAT.-T-.A .........G A.T.------
Psalmopoeus     A.GCT.C.C. TTT.G.AAG. .....A.AA. .A..C-T-.A .........T CAT-------

                                                                                                 120
Ryuthela        TGTGCTAAGG TAGCATAATC ATTTGTCTTT TAAATGAGGT CTGGAATGAA GGGTTTGATC
Dolomedes       .......... .......... .......... ...T.A.A.A ..A...CA.. A.A...A.-.
Pisaura         .......... .......... .......... ...T...A.A ..A...CA.. A.A...A.-.
Pardosa         .......... .........A .......... ...T.A.A.A ..A...CA.. A.....A.-.
C.salei         .......... .........A .......... ...T.A.A.A ..A...CA.. A.A...A.-.
C.getazi        .......... .........A .......... ...T.A.A.A ..A....A.. A.A...A.-.
C.coccineus     .......... .........A .......... ...T.A.A.A ..A...CA.. A.A...A.-.
Phoneutria      C......... ...T.....A .C........ ...T.A.A.A .-AA..CA.. A.A...A.-.
Clubiona        A......... .......... ...C...... ...T...A.A ..A...CA.. A.A..AA.-.
Psalmopoeus     .......... .....C...A .A.A.C..A. ...T..TA.G A...C..... A...C.A.-.

                                                                                                 180
Ryuthela        GAAGAAAGTC CTGTCTCTTT ATTATTTGGT -GAATTAAAT TAGCTAGTAA AAAGGCTAGT
Dolomedes       ATTT...T.A A.T.T.TAAA TC..A..AT. TA.....TT. ..AA.-.... ...AA.ATT.
Pisaura         TTTT...T.A A.A.T.TAAA T....AATT. TA.....TC. ..AA.-.... ....A.ATT.
Pardosa         ATCTC..T.A A.A.T.TA.A .GA.AC.TT. TA.....TT. ..AT.-.... ...AA.A.T.
C.salei         ATCTT..T.T A.T...TAAA T....C.AT. TA.....TC. ..AA.-.... ....A.ATT.
C.getazi        ATTTT..T.T A.T.T.TAAA T....A.AT. CA.....TC. ..AA.-.... ....A.ATT.
C.coccineus     ATCTT..T.T A.T.T.TAAA T....C.AT. TA.....TC. ..AA.-.... ....A.ATT.
Phoneutria      ATTTT...A. A...AA-ACA ....CA.AT. TA..C..TT. ...T.TT.C. ...AAAA.C.
Clubiona        ATTTT..T.A T.T.A.T..A .A..A..AT. AA....TTCC ..AA.-.... ....A.ATT.
Psalmopoeus     ATGA.GCT.T ..T.A.TA.A .AA..GAAT. GA...T.GCA .GAAA-.... ....A..TTA

                                                                                                 240
Ryuthela        ATAGGCCTGA AAGACGATAA GACCCTATTA AGCTTAATTT TTAAAATTTT ACTGGGGCGG
Dolomedes       ...TTATA.. .......... ........CG .A...TTAC. ...G--..AA .......TA.
Pisaura         ...ATTAA.. .......... .........G .A...T-AC. ...G--...A .......TA.
Pardosa         T.TAAAAA.. .......... ........CG .A...T-AC. ...G--...A .......AA.
C.salei         ..CTAATA.. .......... ........C. .A...T-AC. ...G--..AA ........A.
C.getazi        ..CTAATA.. .......... ........C. .A...T-AC. ...G--..AA ........A.
C.coccineus     ...TAATA.. .......... ........C. .A...T-AC. ...G--..AA ........A.
Phoneutria      ...CAAAAA. .......C.. .........G .A....-AC. ...G--..CA ........A.
Clubiona        .C.ATATA.. .......C.. .........G .A....-AC. A.TG--..AA ........A.
Psalmopoeus
```

Figure 2.—Multiple alignment of the mt16S rDNA sequences of the nine spider species investigated. Periods represent nucleotide identity with the reference sequence *Ryuthela nishihirai*. Dashes indicate positions where gaps were introduced to obtain maximal alignment. Highly conserved sequences in mt16S rDNA (as marked in Fig. 3) are underlined. *Psalmopoeus* sp., a theraphosid spider is included in this figure but was only partially sequenced and not taken into further consideration in this paper.

method of Sanger et al. (1977) using a Sequenase kit (U. S. Biochemical) as described in the protocol of the manufacturer. Sequencing was performed in both directions with the primers also used for the PCR. Sequencing reactions were electrophoresed on 7.5% polyacrylamide gels for 2–7 h (Sambrook et al. 1989).

Data analysis.—Sequence data were aligned

```
                                                                                300
Ryuthela        TAGGATAAGA TTATAATCTT ATCCATAATG GTTGATATTT ATTGACCCAA TTTTATTGAG
Dolomedes       .TAAT...-- ....T..T.. .ATTTA-.AT AAAATCT.AA TAA....T.. .A.A...A.T
Pisaura         .TAAT...-- ....T..T.. .ATA.A-.AT AA.TT..AAA T.C....T.. ...A...A.T
Pardosa         .TAAT...-- ....C..T.. .AT-.....T AAAT...CAA T......T.. .AC....A.C
C.salei         .TAAT...-- ......CT.. .ATT..T..T AAATTC..AA -.A....T.. .A.A...A.T
C.getazi        .TAAT...-- .......T.. .ATT..T.AT AAATT.GAAA T.A....T.. .A.A...A.T
C.coccineus     .T.AT...-- .......T.. CATT..T.AT .AATT.CAAA C.A....T.. .A.A...A.T
Phoneutria      .T.AT...-- AA..-..... .ATT.C.T.T TAA--C.AAA TA.....T.. .CCA...A.T
Clubiona        .TAA....-- ...AT..T.. ..TT.CT..T AA.T.ATAAA T---.T.T.. .A.....A.T
Psalmopoeus                                                 TTTA.C. .C..CGATTA

                                                                                360
Ryuthela        GGTAAGATAA AGCTACTATA GGGATAACAG CTTAATTTTC CTTTGAAGAT CTTATTTATT
Dolomedes       TTC.TA..C. ..T...CG.. .......... .G....AAAA T.C.T..... ........AA
Pisaura         TAA.TA..T. ..T...C... .......... .G.....AAA T...TT.... .......GAA
Pardosa         AA..TA..T. ..T...CG.. .......... .G....AAAA T..CT..... ........AA
C.salei         TA.TTA..C. ..T....G.. .......... .G....AAAA T...A..... ......A.AA
C.getazi        TACTTA..T. ..T....G.. .......... .G....AAAA T...A..... ......A.AA
C.coccineus     TAATTA..C. ..T....G.. .......... .G....AAAA T...A..... ......A.AA
Phoneutria      TAA..A..C. ..T...C... .......... .G....AA.T A.C.A...C. ..C..AC.AA
Clubiona        AAA------. ..T...C... .......... .A....AAAT A...A..... .....AC.AA
Psalmopoeus     TAAT.C.CC. ..T...CGC. .......... .AC...C..T T.CAAG...C .....CC.AA

                                                                                420
Ryuthela        GGAAAGTTTG AGACCTCGAT GTTGAATTAA AGTACCTTAT AGGCGCAGTA GGCTA-TAAA
Dolomedes       AT....A... C......... .....T.... TAA-...A.. TCA....A.. .TAA...T..
Pisaura         ATTT..A... C......... .....T.... TT.-...A.. TTA....A.. .TT....T..
Pardosa         AT....A... C......... .....T.... TAA-...A.. TAA....A.. .TTA...T..
C.salei         AT....A... C......... .....T.... TT.T...A.. TAA....A.. .AAA...T..
C.getazi        AT....A... C......... .....T.... TT.T...A.. TTT....A.. .AAA......
C.coccineus     AT....A... C......... .....T.... TT.T...A.. TAA....A.. .AGA...T..
Phoneutria      AA.T..A... C......... .......... TAAT.-.A.. TT.....A.. .CAA..A...
Clubiona        TAT..TA..A T......... .......... TAA-...ATA TTAT...A.. ATTAT.A...
Psalmopoeus     AA....A..A T......... .......... -.ATT..CC. TAAA...AAG CTTA.GA...

                                                  446
Ryuthela        GGAAGTCTGT TCGACTTTTA AATCTT
Dolomedes       .......... .......... ..AAA.
Pisaura         .......... .......... ..AAA.
Pardosa         .......... .......... ..AAA.
C.salei         .......... .........G ...AA.
C.getazi        .......... .......... ...AA.
C.coccineus     .......... .......... ...AA.
Phoneutria      .A........ .......... ..AAA.
Clubiona        .......... .......... ..AAA.
Psalmopoeus     .A........ .......... ..C.-.
```

by CLUSTAL V (Higgins et al. 1991). Pairwise alignment and calculation of percent differences was carried out by MICROGENIE (Queen & Korn 1983). The data was subjected to DNAPARS and DNABOOT of PHYLIP 3.4 (Felsenstein 1991). Gaps comprising more than one site were treated as missing data, and thus played no role in phylogenetic reconstruction.

Aligned sequences were fitted into available secondary-structure models from Gutell & Fox (1988).

RESULTS

Initially we tried to use the conserved primers of Kocher et al. (1989) to amplify a fragment of the cytochrome b gene of spiders. These primers

Table 2.—Percentage of base identities of the mt16S rDNA between the investigated spiders. The values are rounded off.

	Ryu.	*Phon.*	*Club.*	*Pard.*	*Pisa.*	*Dolo.*	*C.coc.*	*C.get.*
Cupiennius salei	66	79	80	87	86	90	97	96
Cupiennius getazi	67	79	80	86	88	90	96	
Cupiennius coccineus	65	79	80	87	88	89		
Dolomedes	67	81	82	88	90			
Pisaura	68	78	81	87				
Pardosa	67	79	79					
Clubiona	65	77						
Phoneutria	64							

proved to be unsuitable for our experimental animals, however. In a second attempt we used primers designed according to those used by Cunningham et al. (1992) to amplify and study mt16S rDNA in crabs. This approach led to the amplification of some fragments but suffered from the poor reproducibility of the results. We therefore chose to apply a two step amplification procedure. In a first step, PCR amplification was performed with simultaneous radioactive labelling of the polymerized DNA. The products were separated on polyacrylamide gels and autoradiographed (Fig. 1). In a second step DNA material of the major amplification product (about 500 bp, see Fig.1) was gel-extracted and subjected to a non-radioactive PCR amplification. A unique distinct band was obtained which was subjected to dideoxy sequencing after gel-extraction (see Methods).

Some bands of the expected size were also eluted from the first experiment. The DNA obtained from them was partially sequenced after reamplification and the identity of its main band with mt16S rDNA confirmed.

The DNA sequences of the nine spider species investigated in this study are shown in Fig. 2. The length of the sequenced fragment varies between 421 (*Clubiona pallidula*) and 444 bases (*Ryuthela nishihirai*). The percentage of identities in sequence is 64% or higher in all pairwise comparisons (Table 2); and a full alignment was reached, assuming a few small deletions only (Fig. 2). This clearly indicates the homology of the sequences determined.

In four cases, two individuals of the same species were examined (*cf.* Table 1) and as expected from previous work on tetragnathid spiders (Croom et al. 1991) no intraspecific variation was found. Interspecific variation between the three *Cupiennius* species is low (3–4%), whereas intergeneric differences vary conspicuously, ranging from 10–36% (Table 2).

The percentage of A and T along the sequenced DNA fragment is high (75.0–78.6%) in all species investigated except the "primitive" liphistiomorph spider *Ryuthela nishihirai* (66.7%). Similar results were obtained by Cunningham et al. (1992) for Crustacea (the "primitive" *Artemia salina*: 63% AT; the highly evolved king crabs *Pagurus* spp.: about 73% AT). In insects data is only available for highly evolved species such as *Drosophila yakuba* (Clary & Wolstenholme 1985) and *Aedes albopictus* (HsuChen et al. 1984). Both of these insects have a very high percentage of AT (about 76%), too. Possibly, an increase in the percentage of A and T is a general trend in arthropod phylogeny (*cf.* Clary & Wolstenholme 1985).

Figure 3 presents an attempt to fit the partial sequence of *Cupiennius salei* rDNA into a generally accepted secondary structure model for animal mt16S rRNA (Gutell & Fox 1988). The resulting secondary structure is very similar to that of the mt16S rRNA of both *Drosophila yakuba* and *Aedes albopictus* which are the only arthropods studied in this regard. In *C. salei* generally well conserved sequences (marked in Fig. 3) take the same positions relative to the overall secondary structure of mt16S rRNA. In addition these sequences show no variation among the nine spiders examined (*cf.* Fig. 2). Thus we conclude that a fragment of mt16S rDNA indeed has been sequenced.

One of the several most parsimonious trees constructed by DNAPARS is shown in Fig. 4. When changing the order of the DNA sequences in the input file the same result was obtained in most cases. Other trees varied slightly regarding the relationships between *Cupiennius*, *Pardosa* and the pisaurids. In no case was *Phoneutria*

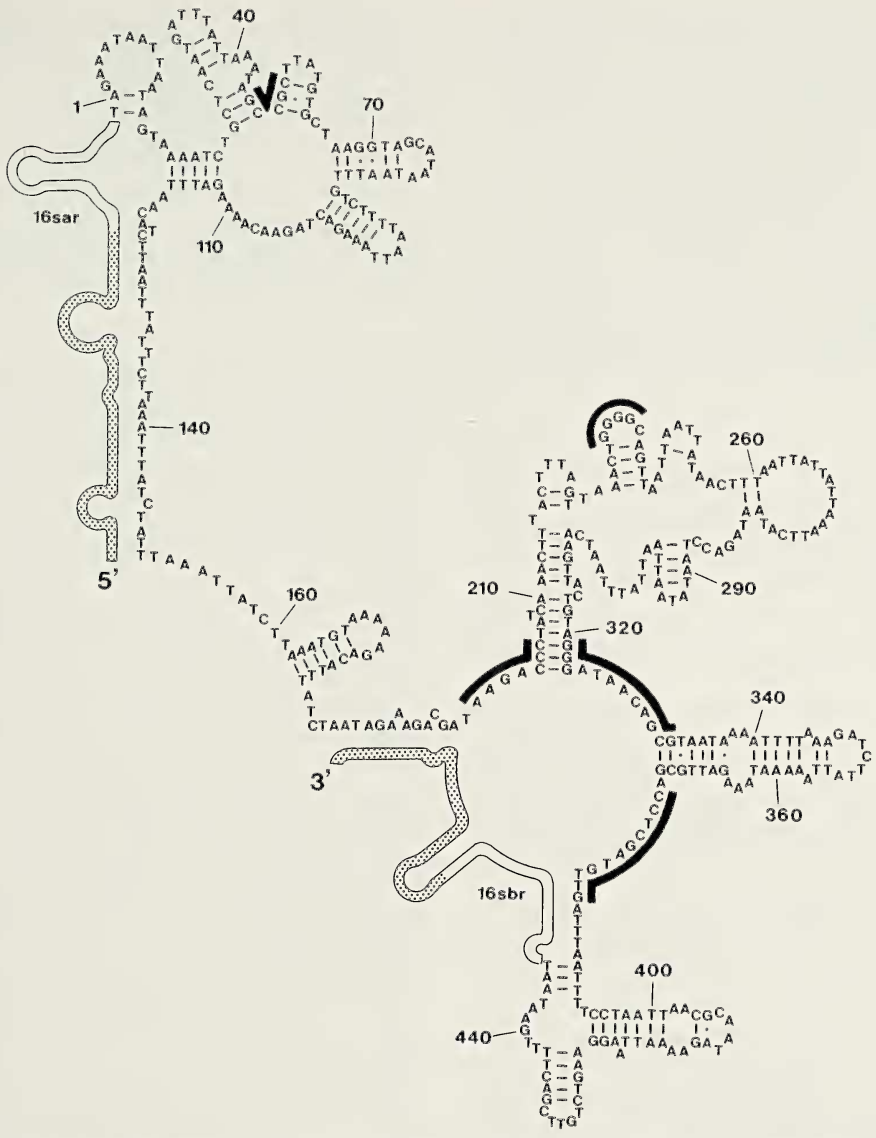

Figure 3.—Mitochondrial DNA of *Cupiennius salei* folded to show the secondary structure of the sequenced 16S ribosomal RNA for which it codes. Dashes represent Watson-Crick pairings, dots represent the weaker hydrogen bonds between T and G or A and G. Grey beams indicate the supposed secondary structure of the adjacent sequences, based on the model for *Drosophila yakuba* in Gutell & Fox (1988). 16sar, 16sbr (white beams): priming sites. Black lines indicate extremely conserved regions known to be almost identical in vertebrates as well as in *Escherichia coli*. Numbering as in Fig. 2.

interpreted as a sister group of *Cupiennius*. However, in a bootstrap analysis with 1000 replicates the three *Cupiennius* species were regarded monophyletic in 94% of the bootstrap estimates. This occurred only 19–54% in the other groups.

DISCUSSION

This communication presents the first extended analysis of spider mt16S rDNAs for phylogenetic studies. It is based on the PCR amplification of this gene fragment in nine spider species representing five families.

The similarity (64–97% identity) of the 421–444 bp long sequences in all cases indicates that homologous sequences have been determined from all nine species. Furthermore, the derived RNA sequences fit well into the conserved secondary structure of other animal mt16S rRNAs

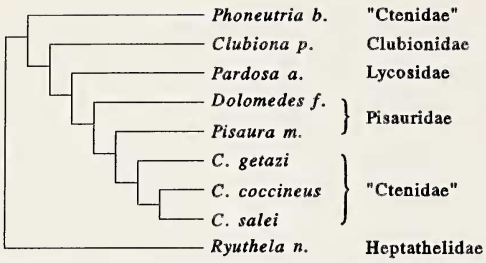

Figure 4.—Mt16S rDNA gene-tree based on the most parsimonious tree obtained in most cases when applying the DNAPARS program of PHYLIP 3.4 (Felsenstein 1991). Note the position of *Phoneutria* in relation to *Cupiennius*.

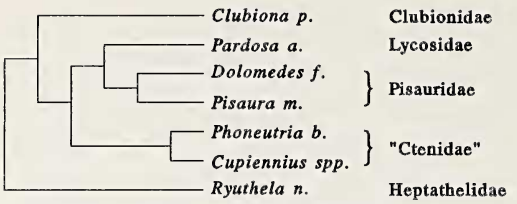

Figure 5.—Current view on phylogenetic relationships of the examined taxa, based on the cladistic hypothesis of Coddington & Levi (1991).

and highly conserved signature sequences of mt16S rRNA can be identified unambiguously. Finally, short sequences of this mt16S rDNA fragment and of a previously determined mt12S rDNA fragment (Croom et al. 1991) are successfully used as primers for the PCR amplification of large, adjacent parts of both rDNAs (R. J. Felber, pers. comm.).

Sequence variation is low when the three species of *Cupiennius* are compared (3–4%) whereas it varies between 10–23% among the Opisthothelae studied here (Table 2). This indicates that sequences of the mt16S rDNA may be useful for studies of spider phylogeny at the family and higher taxonomic levels.

Although limited, the data presented here reveal an intriguing result: In all of the maximum parsimonious gene trees *Phoneutria* was more distantly related to *Cupiennius* than *Pardosa* and the two pisaurids. This sheds doubt on the available classification of both genera—*Phoneutria* and *Cupiennius*—within the Ctenidae and indeed on the monophyly of this family, which was established by Keyserling in 1877. The monophyly of the included genera has mainly been based on the following morphological characters: (1) ecribellate spiders with (2) an eye-formula quite peculiar to them among (3) the two-clawed spiders (Pickard-Cambridge 1897; Bücherl et al. 1964). These are all characters which also occur in other families and therefore cannot be considered as strong synapomorphies. Depending on the position of the nominal genus *Ctenus*—which could not be investigated in this project—either a new family for *Cupiennius* (*Ctenus* closely related to *Phoneutria*) or for *Phoneutria* (*Ctenus* closely related to *Cupiennius*) may well be appropriate. One obvious next step is therefore the examination of the nominal species of the Ctenidae.

The critical evaluation of this far-reaching implication of our study asks for answers to several questions. One of these concerns the identification of *Phoneutria* species, which is still a problem. Many Neotropical taxa are still undescribed (Coddington & Levi 1991) and there is no recent revision of the genus. The two female individuals studied by us were identified using the key of Bücherl (1969). Their genitalia were dissected, treated with KOH and then compared with illustrations provided by Schiapelli & Gerschman (1973) and by Valerio (1983). Given the species specificity of spider genitalia, our morphological study makes it highly probable that the spiders in question belong to the species *Phoneutria boliviensis*. Another question is the possibility of contaminations. However, DNA was extracted from both individuals independently. Separate amplification and up to threefold sequencing of some segments led to absolutely identical results. Contaminations are therefore considered a very unlikely reason for the surprising position of *Phoneutria* in the gene tree derived from our DNA analysis.

Except for the apparent polyphyly of the Ctenidae, the maximum parsimony tree proposed in Fig. 4 does not allow any further conclusions on spider phylogeny. Details such as the relationships between the three *Cupiennius* species or between *Pisaura* and *Dolomedes* should not be given too much weight. According to the low values obtained by bootstrap analysis these relationships are not significant (there is significant evidence for the monophyly of a group if it occurs in at least 95% of the bootstrap replicates: Felsenstein 1991). From a morphological point of view *Phoneutria* belongs to the monophylum of spiders characterized by at least one pair of secondary eyes with a grate-type tapetum (Homann 1971). *Clubiona* (with a canoe-shaped tapetum) on the other hand does not belong to this monophylum and should therefore branch off deeper

in the tree (Fig. 5). There is not sufficient data to resolve this discrepancy, however. We now rather need a comprehensive examination of additional genera, including both molecular and morphological information.

ACKNOWLEDGMENTS

Financial support by the Austrian Science Foundation (FWF; P7896 B) to F. G. B. is highly acknowledged.

LITERATURE CITED

Bücherl, W., S. Lucas & V. Dessimoni. 1964. Aranhas da familia Ctenidae, Subfamilia Ctenidae. I. Redescricao dos generos *Ctenus* Walckenaer 1805 e *Phoneutria* Perty 1833. Mem. Inst. Butantan, 31: 95–102.

Bücherl, W. 1969. Aranhas da família Ctenidae. II Phoneutriinae subfamília nova. Mem. Inst. Butantan, 34:25–31.

Clary, D. O. & D. R. Wolstenholme. 1985. The mitochondrial DNA molecule of *Drosophila yakuba*: Nucleotide sequence, gene organization, and genetic code. J. Mol. Evol., 22:252–271.

Coddington, J. A. & H. W. Levi. 1991. Systematics and evolution of spiders (Araneae). Annu. Rev. Ecol. Syst., 22:565–592.

Croom, H. B., R. G. Gillespie & S. R. Palumbi. 1991. Mitochondrial DNA sequences coding for a portion of the RNA of the small ribosomal subunits of *Tetragnatha mandibulata* and *Tetragnatha hawaiensis* (Araneae, Tetragnathidae). J. Arachnol., 19:210–214.

Cunningham, C. W., N. W. Blackstone & L. W. Buss. 1992. Evolution of king crabs from hermit crab ancestors. Nature, 355:539–542.

Felsenstein, J. 1991. Manual to PHYLIP (Phylogeny Inference Package) Version 3.4.

Fernholm, B., K. Bremer & H. Jörnvall. 1989. The hierarchy of life. Molecules and morphology in phylogenetic analysis. Pp. 1–499. Excerpta Medica, Amsterdam - New York - Oxford.

Gatesy, J., D. Yelon, R. DeSalle & E. S. Vrba. 1992. Phylogeny of the Bovidae (Artiodactyla, Mammalia), based on mitochondrial ribosomal DNA sequences. Mol. Biol. Evol, 9:433–446.

Gutell, R. R. & G. E. Fox. 1988. A compilation of large subunit RNA sequences presented in a structural format. Nucleic Acids Res., 16 :R175–R313.

Higgins, D. G., A. J. Bleasby & R. Fuchs. 1992. CLUSTAL V: improved software for multiple sequence alignment. CABIOS, 8:189–191.

Homann, H. 1971. Die Augen der Araneae. Anatomie Ontogenie und Bedeutung für die Systematik (Chelicerata, Arachnida). Z. Morph. Tiere, 69:201–272.

HsuChen, C. C., R. M. Koten & D. T. Dubin. 1984. Sequences of the coding and flanking regions of the large ribosomal subunit RNA gene of the mosquito mitochondria. Nucleic Acids Res., 12: 7771–7785.

Keyserling, E. 1877. Über amerikanische Spinnenarten der Unterordnung Citigradae. Verh. zool.-bot. Ges. Wien, 26:609–708.

Kocher, T. D., W. K. Thomas, A. Meyer, S. V. Edwards, S. Pääbo, F. X. Villablanca & A. C. Wilson. 1989. Dynamics of mitochondrial DNA evolution in animals: Amplification and sequencing with conserved primers. Proc. Natl. Acad. Sci. USA, 86:6196–6200.

Lachmuth, U., M. Grasshoff & F. G. Barth. 1985. Taxonomische Revision der Gattung *Cupiennius* Simon 1891. Senck. Biol., 65:329–372.

Lehtinen, P. T. 1967. Classification of the cribellate spiders and some allied families, with notes on the evolution of the suborder Aranaeomorpha. Ann. Zool. Fennici, 4:199-468.

Mullis, K. B. & F. A. Faloona. 1987. Specific synthesis of DNA in vitro via a polymerase catalyzed chain reaction. Methods in Enzymology, 155:335–350.

Pickard-Cambridge, F. 1897-1905. Arachnida, Araneida and Opiliones. Biologia Centrali-Americana, 2:1–610. London.

Queen, C. & L. Korn. 1983. Manual to MICROGENIE (TM) sequence analysis program.

Saiki, R. K., D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis, & H. A. Erlich. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science, 239:487–491.

Sambrook, J., T. Fritsch & T. Maniatis. 1989. Polyacrylamide gel electrophoresis. Pp. 6.36–6.48, *In* Molecular Cloning, 2nd ed., vol. 1. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Sanger, F., S. Nicklen & A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA, 74:5463–5467.

Schiapelli, R. D. & B. S. Gerschman de Pikelin. 1972. Diagnosis de *Phoneutria reidyi* (F. O. Pickard-Cambridge, 1897) y de *Phoneutria boliviensis* (F. O. Pickard-Cambridge, 1897) (Araneae, Ctenidae). Rev. Soc. Ent. Argentina, 34:31–38.

Valerio, C. E. 1983. Sobre la presencia de *Phoneutria boliviensis* (F. O. Pickard-Cambridge) (Araneae, Ctenidae) en Costa Rica. J. Arachnol., 11:101–102.

Wilson, A. C., R. L. Cann, S. M. Carr, M. George, U. B. Gyllensten, K. M. Helm-Bychowski, R. G. Higuchi, S. R. Palumbi, E. M. Prager, R. D. Sage & M. Stoneking. 1985. Mitochondrial DNA and two perspectives on evolutionary genetics. Biol. J. Linn. Soc., 26:375–400.

Manuscript received 11 January 1993, revised 2 June 1993.